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Variation in female mating behavior and success in the damselfly,

Calopteryx maculata

Suzanne E. Allison

A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Master of Science

Department of Biology

December 2016

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Acknowledgments

I would like to thank my thesis advisor, Dr. Idelle Cooper, and the members of my committee, Dr. Heather Griscom and Dr. Reid Harris, for their valuable guidance and support during this project. This study would not have been possible without the superb assistance of my fellow lab members, Benjamin Colligan and Phoebe Cook. I am also grateful for the extra field assistance provided by Amber Alexander, Cecilia Colligan, Gillian Slekar, Dakota Kobler, and Katelyn Walters.

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Abstract

Traditionally, the study of sexual selection has focused on the evolution of elaborate male traits and how they enhance the ability to out-compete other males directly (access to females) and indirectly (access to desirable territories or resources). Female trait studies have focused most on evolved preferences for male traits. While we know much about how sexual selection acts on males, there is a deficit of equivalent study on females. In insects, including damselflies, male size and pigmentation are positively correlated with fat reserves and immune abilities, and therefore with male competitive ability. Here, we show that phenotypic variation that has been well-documented in males of the Ebony jewelwing damselfly, *Calopteryx maculata*, is also present in females of the species. We measured female mating success and behavior of *C. maculata* at Smith Creek in Rockingham County, Virginia. Males were marked with multiple colors of fluorescent powder that was transferred to females when mating. Uniquely-numbered females were digitally scanned and repeatedly observed throughout the summer. We determined that there is significant variation in female mating frequency, wing pigmentation, size, and shape. The study of trait variation within females, and thus the opportunity for selection to act on those traits, is essential in understanding how evolution on females may have contributed to sex differences, and may change the way we think about the role of females in sexual selection.

I. Introduction

Darwin (1871) first proposed that the elaborate phenotypic traits of males in many closely related species were the result of competition between males for access to mates and female choice of mates. Despite initial resistance and ongoing debate, this theory of sexual selection is generally accepted (Wallace 1889; Huxley 1938; Mayr 1972; Dall et al. 2006; Shuker 2010; Hosken and House 2011). However, recent studies indicate that the roles of males and females in sexual selection may not be as dichotomous as generally portrayed (Clutton-Brock 2007, 2009). Because most studies have focused on competition in males and choice in females, other roles of the sexes, particularly females, have not been examined very thoroughly. Here, I will quantify the variation in female mating behavior and success in a frequently-used system in the study of sexual selection, the Ebony Jewelwing damselfly, *Calopteryx maculata*.

The basis of the sexual selection hypothesis is that a male may maximize his reproductive success by mating with as many females as possible, while a female's reproductive success depends more upon the quality of her mate (Fisher 1930; Bateman 1948; Trivers 1972; Andersson 1994). Because sperm is usually not as limited as eggs, a male may increase his reproductive success through direct or indirect competition with other males for access to females. Additionally, a successful male may have display traits that are preferred by females directly. Consequently, the combination of male-male competition and female preference for specific male traits can drive the evolution of sexually-selected traits.

More recently, sexual selection has been given the more inclusive definition of "competition for mates" (Clutton-Brock 2009; Shuker 2010; Rosvall 2011), allowing for the

consideration of competition and preferences of *both* sexes, not just competition by males and preference by females. When the definition of sexual selection is broadened so that it includes traits likely to occur in both males and females, it becomes apparent that the majority of information currently available is inadequate to understand sexual selection because it primarily focuses on variation in male traits related to male competition and female choice (Shuster and Wade 2003; Hosken and House 2011; Rosvall 2011). The few studies that have addressed variation in female reproductive success have demonstrated that measuring male reproductive success alone may not be telling the whole story of sexual selection because in many cases there has been more variation in females than previously thought (Siva-Jothy et al. 1995; Fincke 1997; Simmons 2001; Cordoba-Aguilar and Cordero-Rivera, 2005; Svensson et al. 2005; Andersson and Simmons 2006; Hosken & House 2011; Cain and Ketterson 2011; Rosvall 2011). By including female variation within the scope of sexual selection, we may broaden our understanding of the evolution of sex differences.

Odonates (damselflies and dragonflies) have been used frequently as research subjects since the 1950s and have proved to be excellent model organisms for the study of evolution, ecology, and behavior due to their widespread distribution, conspicuous behavior, ease of observation in natural habitats, site fidelity of adults, and ease of visually distinguishing between sexes (Corbet 2004; Cordoba-Aguilar and Cordero-Rivera 2005). Many interesting physiological and behavioral traits of odonates suggest that females play an important role in sexual selection. For example, in many damselfly species, there is a delay between copulation and fertilization, which can enable post-copulatory control of fertilization. Females have a bursa copulatrix, which receives and stores sperm, and one or two spermathecae, which are used to store sperm until she is ready to oviposit. Because of a female's ability to store sperm, she is more able to control which male ultimately fertilizes

her eggs, even if she mates multiple times. This cryptic female choice scenario has led to a number of counter-adaptations in both males and females, which illustrate some of the conflicts surrounding sexual reproduction (Uhlir and Cordero Rivera 2005; Waage 1979). For example, some males are able to cause a rival's sperm stored in the inaccessible spermathecae to be ejected by stimulating sensory structures in the females (Cordoba-Aguilar et al. 2003). Multiple studies have examined the role of cryptic female choice, and how pre-, syn-, and post-copulatory behavior by males may influence females' decisions (Uhlir and Cordero Rivera 2005; Eberhard 1996).

Many studies of *Calopteryx* damselflies have examined which male traits females prefer during mate choice (Eberhard 1996; Siva-Jothy 1999; Cordoba-Aguilar et al. 2003). Darker wing pigmentation is positively correlated with male mating success, presumably because a higher amount of pigment is associated with larger body size, fat reserves, and stronger immune abilities, which allows them to more successfully obtain and defend a territory (Siva-Jothy 1999). Males defend territories along slow-moving streams and rivers and perform courtship displays, such as hovering before perched females, and the "cross display", where a male perches near an oviposition site, raises his abdomen to an angle of 45 deg. or more with hindwings spread forward at an angle of approximately 45 degrees and forewings vertically folded over the back (Waage 1975). These male courtship behaviors seem to call attention to their wings, and females are thought to choose larger males with higher amounts of wing pigmentation, as well as those males with territories containing the best sites for oviposition within submergent vegetation (Siva-Jothy 1999). Obviously, insect wings are not only important in communication, but also affect an individual's ability to survive and reproduce through flight (West-Eberhard 1984; Grimaldi and Engel 2005; Jones et al. 2013). These multiple functions of wings lead to multiple selection pressures (Grimaldi

and Engel 2005; Outomuro et al. 2012). Wing traits that optimize flight are typically considered to be under natural selection, while those associated with ornamentation and signaling are expected to be under sexual selection. Congruently, studies in other *Calopteryx* damselflies have shown forewings to be more associated with flight ability and hindwings with display (Outomuro et al. 2012, 2013).

In this study, I hypothesized that females would vary in mating success more than previously thought, which is to say that there is some variation, and that this variation would be correlated with phenotypic traits. To quantify variation in traits related to female reproductive success, I measured mating frequency, phenotypic traits (wing pigmentation, size, shape), and reproductive behavior (courtship, copulation, oviposition), in females of the damselfly *C. maculata* at Smith Creek in Rockingham County, Virginia. Because of the traits under sexual selection in male *Calopteryx* damselflies, I predicted that wing pigmentation and wing size would be greater in females that mated more frequently than others, and that these traits would vary more on hindwings than forewings.

II. Materials and Methods

Study Site

We selected an area of damselfly habitat in which we could realistically capture and mark almost all of the damselflies in a single day, a 2,500 m² area encompassing Smith Creek and its banks in Rockingham County, Virginia (38.512129 N, -78.744936 W; Fig. 1).

Sampling took place between June 5 and August 2, 2015. Individual damselflies were netted between 9:00 a.m. and 5:00 p.m., unless it was raining, which is the time during which they are active.

Female mating frequency

In order to quantify female mating success, we powdered males on their terminal claspers with one of four different colors of fluorescent powder, which would transfer to females when initiating mating because males use their claspers to grab females around the neck. By observing the number of different powder colors transferred to an individual female, we could determine the minimum number of times she had mated since her last capture. Four different colors (blue, green, orange, pink) were the most that we could distinguish between easily, so we could only identify whether a female mated at least four times. Our estimate is conservative because females could have mated with unmarked males or mated with males of the same color as previous mates. We confirmed that an equal number of males were marked with each color, and that females showed no bias toward a particular powder color (Table A1). On each sampling day, all male damselflies seen in the study area were captured, powdered, and released. If a previously marked male was recaptured, the same color powder was reapplied. We marked a total number of 1,050 unique males during the study period.

In order to determine the amount of variation in mating frequency, we followed individual female damselflies over time. Every female captured was given a unique number code using water-based paint pens on the underside of the abdomen before release. We marked 415 unique females. Each marked female was checked for the presence of fluorescent powder at the back of the head, where males made contact when joining in tandem prior to copulation. An ultra-violet light in a black box was used to increase the visibility of the powder, which allowed us to see even a single grain of powder on a female. Based on our recapture data, the powder remained visible on females for approximately two weeks. If we detected any powder on parts of the female other than the area where she was grasped by a male, we did not include it in our count because of the possibility that it may have been acquired through contact other than mating.

To measure wing pigmentation, shape, and size, we obtained digital images of females by placing each live damselfly on a portable flatbed scanner. Transparent microscope slides were placed over the wings in order to immobilize the damselfly during scanning. A color standard and measurement scale were included in each scan, and the scanner covered with light-blocking fabric to ensure light and color were consistent in each image. The damselflies were scanned at a resolution of 600 dpi. This method did not damage the females, which flew away after being scanned and were often recaptured in good condition.

I used a categorical age designation to get a relative estimate of condition and life stage because it was difficult to know an exact age by following an individual damselfly from emergence. Age was estimated as one of the following five ranks: 0 = very recently emerged (teneral), soft cuticle, pale coloration; 0.5 = cuticle less soft, wings fragile, less pale in color; 1

= mature adult, cuticle hardened and shiny, no damage or wear visible, color fully developed; 1.5 = cuticle still mostly shiny, little-to-no wing damage; 2 = cuticle dull or rough, damage to wings. Because age was a qualitative estimate, the categories of adults deemed as 1.0 and 1.5 were combined into a single group for analysis. Categories 0.0, 0.5, and 2.0 were excluded from analysis, however the total frequency of females in each age category is reported in the appendix (Table A2). Those grouped as 0.0 and 0.5 were unable to be marked or scanned because the cuticle had not yet hardened enough for handling without harm. Those in category 2.0 were excluded because many of the damselflies in that category were damaged and unable to be used in image analysis.

In order to minimize the opportunity for a female to mate with an unmarked male, we marked males in a wider area (approx. 2,500 m²), than the females (approx. 1000 m²; Fig. 1). Data on female mating frequency from the first 24 days of the study are not included in analysis due to the fact it took several days for the male population to be sufficiently marked with powder, and to allow us time to become consistent in our sampling techniques.

Image analysis

The following wing traits were measured in one forewing and one hindwing of each female damselfly using Adobe Photoshop CS6 (version 13.0, 2012): wing hue (degrees), saturation (percent), brightness (percent), pterostigma (white spot at the tip of each wing) hue, saturation, brightness, and area (cm²). Average hue, saturation, and brightness were measured on wings by selecting a 100x100-pixel square of the wing, once on the forewing's center, and twice on the hindwing, both in the center and near the wingtip (Fig. 2). The hindwings were measured in two places because they have more within-wing pigment variation, and this allowed us to capture the contrast between different areas of the wing.

Once an area was selected, the average color values could be measured by applying the “blur, average” filter. We measured this to identify whether it is a visual cue, particularly in contrast with the dark background of the wing. Average pterostigma hue, saturation, and brightness values were obtained in the same manner, except the entire pterostigma was selected for measurement, which also allowed us to measure its area (cm²).

Wing size and shape were measured using the program tpsDig2 (Rohlf 2016) for the placement of digital landmarks on the images, and the program PAST versions 2 and 3 (Hammer et al. 2009) for landmark analysis. On each digital image, I placed 11 landmarks on the right forewing and 11 on the right hindwing (Fig. 2). The landmarks mark homologous points on each wing and were chosen based on previous studies of damselfly wing shape (Outomuro et al. 2012 & 2013).

Statistical analysis

A subset of all female damselflies captured were used in mating frequency analyses (n = 96). Those included met the following criteria: an ultra-violet light in a black box was used to confirm the presence of powder transferred during mating, a clear scan was obtained at a resolution of 600 dpi, the fore- and hindwings on the right side of the body were not excessively damaged, and the female was captured during the period in which the male population was fully marked (6/29 – 8/2/2015).

An analysis of variance (ANOVA) was performed to determine whether female phenotypic traits differed according to their mating success of zero to four mates, conservatively. We also analyzed these data by grouping the females into two groups, unmated and mated, and then performing independent samples t-tests.

We determined the mean centroid size of each wing using PAST v2, which calculates the geometric mean of the distance between each landmark and the centroid point of the polygon formed by all landmarks. In order to measure wing shape, we first aligned the x and y coordinates of the landmarks using a generalized procrustes alignment in PAST v3, which removes the variables of size, position, and orientation in the digital image and allows for measurement of the non-allometric component of the wings. After alignment, the 11 landmarks on each wing were used to obtain principal components that described the variation in wing shape. We used an ANOVA on the first three principal components to determine whether there were differences in mating frequency related to wing shape, and we used a linear regression to determine any relationship between wing shape and hindwing pigment saturation. All analyses were performed using SPSS (version 22), unless indicated above.



Fig. 1. Map of study site.

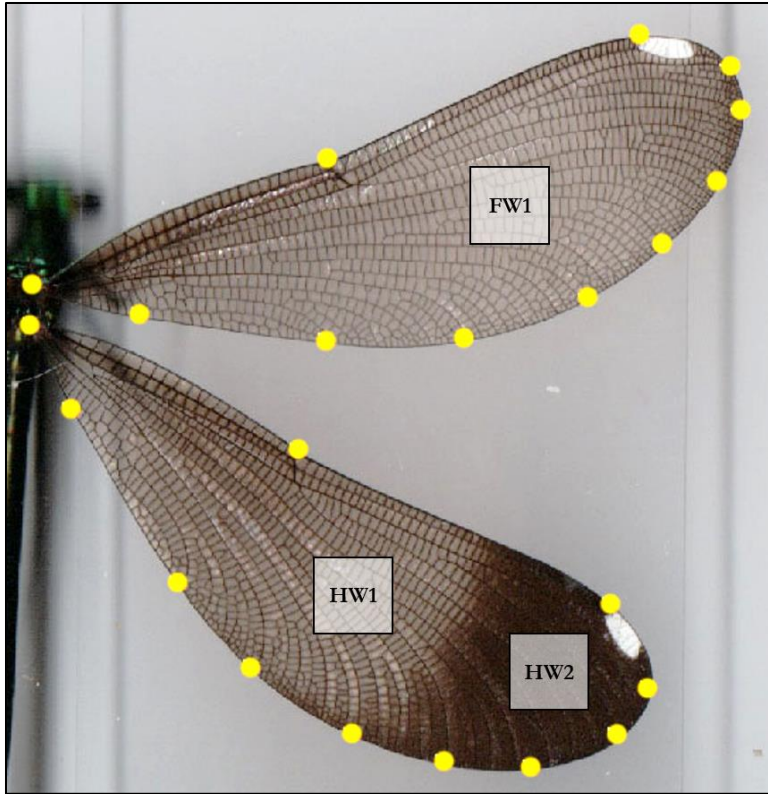


Fig. 2. Example of a scanned female damselfly. The centroid size of each wing was calculated using 11 digital landmarks (in yellow) on a forewing and hindwing. The average hue, saturation, and brightness were measured on wings by selecting a 100 x 100-pixel square of the wing, once on the forewing's center (FW1), and twice on the hindwing (HW1, HW2).

III. Results

Mating Frequency

Out of the total number of females included in the analysis ($n = 96$), 33.3% showed no evidence of mating and approximately 66.7% mated at least once (Fig. 3). This variation was not due to a longer time between captures for the females with more matings, as the mean recapture time was 1.47, $SE = .51$ days for all categories. There was no difference in recapture time between mating frequency categories, Kruskal-Wallis, $H = 5.738$, $df = 3$, $P = 0.125$, $n = 96$.

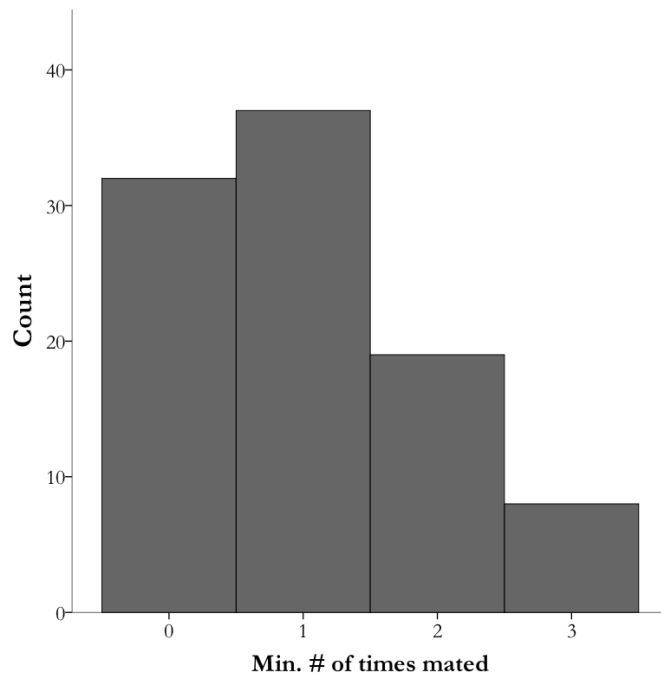


Fig. 3. Distribution of female damselflies grouped by mating number ($n = 96$).

Wing Pigment

Female damselflies that mated at least once had significantly darker wing tip pigmentation (shown as a higher percent saturation), mean sat. = 55.92 %, $SE = 1.174$, than

those that showed no evidence of mating, mean sat. = 50.88 %, SE = 2.056, $t = 2.289$, $df = 94$, $P = 0.024$ (Fig. 4).

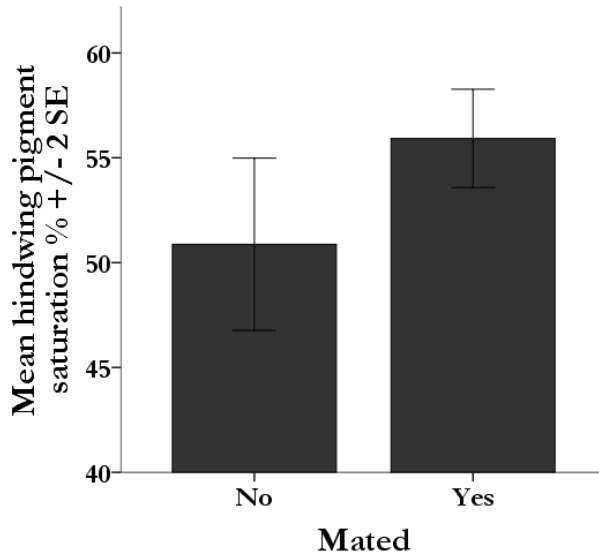


Fig. 4. Females who had mated at least once had darker pigmentation on hindwings compared to those who showed no evidence of mating.

The contrast between pigment saturation of the hindwing center (HW1) and tip (HW2) did not show a difference between mating groups, ANOVA, $F = 2.233$, $df = 3$, $P = 0.090$, $n = 96$ (Fig. 5). However, the contrast between the pterostigma and hindwing tip differed between mating frequency groups. Females that mated more had a greater contrast between the dark tip of the hindwing and light color of the pterostigma, ANOVA, $F = 2.707$, $df = 3$, $P = 0.050$, $n = 96$ (Fig. 6).

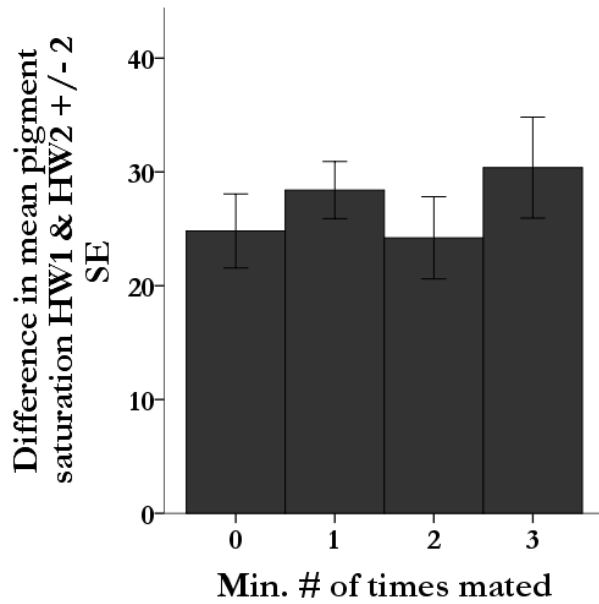


Fig. 5. There was no difference between mating frequency groups in the pigment contrast between the center and tip of hindwings.

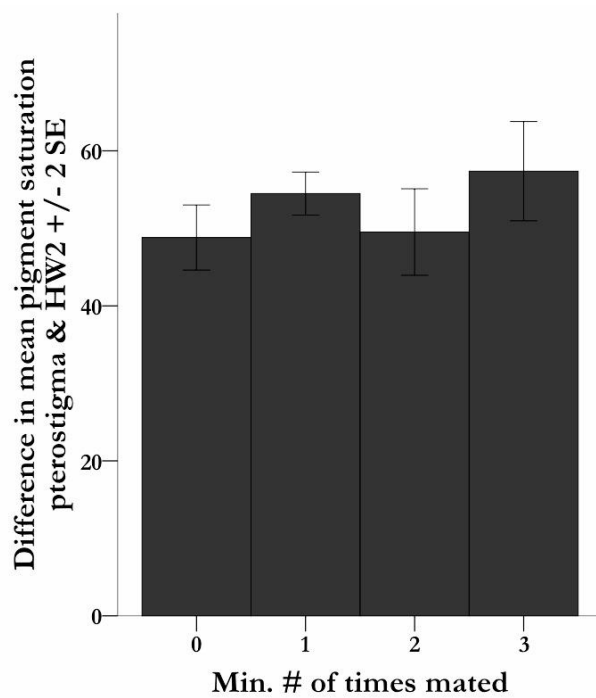


Fig. 6. The contrast in pigment saturation between the pterostigma and hindwing tip was higher in females that showed evidence of mating at least three times.

Wing Size

There was little size variation within fore- and hindwings in females (mean centroid size of forewing = 3.758, SE = 0.013; hindwing = 3.121, SE = 0.012). The forewings of damselflies that mated at least once were significantly larger than those that showed no evidence of mating ($t = 2.053$, $df = 82$, $P = 0.043$; Fig. 7). The hindwings showed no significant difference (mean centroid size mated = 3.136, SE = 0.014; unmated = 3.091. SE = 0.019; $t = 1.893$, $df = 82$, $P = 0.62$; Fig. 7).

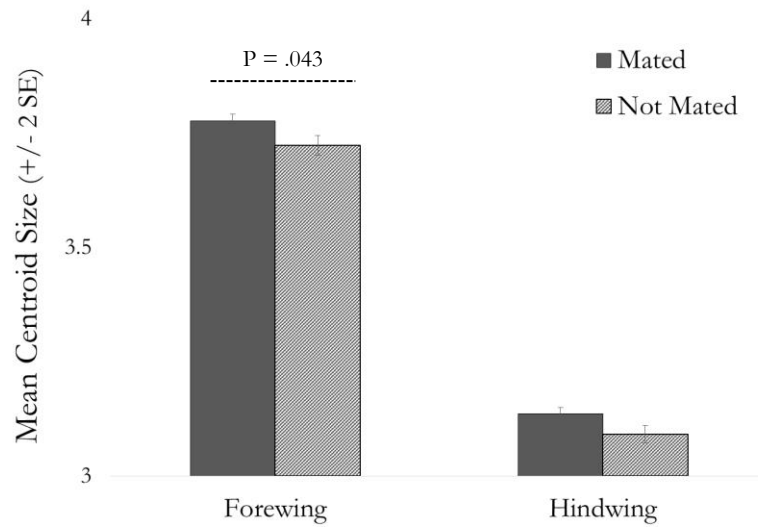


Fig. 7. The forewings of females that had mated at least once were larger than the forewings of those with no evidence of mating. Hindwings showed no difference in size between mating categories.

Wing Shape

There was a difference in the mean principal component 1 scores (PC1) of both fore- and hindwing shape between females that showed no evidence of mating, versus those who had mated at least once, PC1 forewing: $t = -2.136$, $df = 82$, $P = 0.036$; PC1 hindwing: $t = 2.426$, $df = 82$, $P = 0.017$ (Fig. 8).

However, an ANOVA showed no difference in mean PC1, PC2, and PC3 scores of forewing shape between the individual mating frequency groups, PC1: $F = 1.473$, $df = 3$, $P = 0.228$; PC2: $F = 0.3388$, $df = 3$, $P = 0.798$; PC3: $F = 0.539$, $df = 3$, $P = 0.657$; (Fig. A1, appendix). Likewise, there was no difference in mean PC1, PC2, and PC3 scores of hindwing shape between individual mating frequency groups, PC1: $F = 1.651$, $df = 3$, $P = 0.184$; PC2: $F = 0.432$, $df = 3$, $P = 0.731$; PC3: $F = 0.398$, $df = 3$, $P = 0.755$ (Fig. A2).

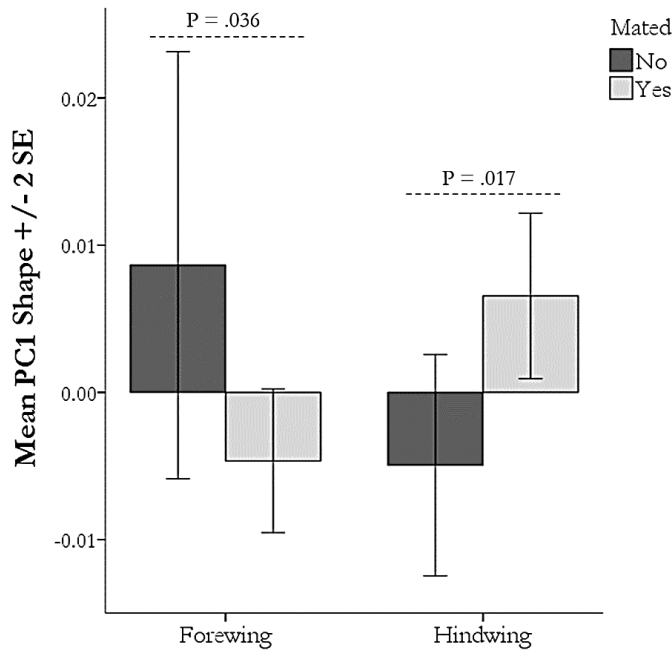


Fig. 8. Both fore- and hindwing shape differed between females that showed no evidence of mating and those that mated at least once.

IV. Discussion

In support of my hypothesis, this study shows that there is more variation in female *C. maculata* mating frequency and behavior than previously appreciated (i.e., that variation does indeed occur), and that mating success is correlated with wing shape, size, and pigmentation. Hindwing pigment was darker in females that mated more, and hindwing shape differed between those that mated at least once and those with no evidence of mating. Forewings were larger in females that mated more frequently, and also differed in shape.

Because one of the most noticeable visual traits limited to female *C. maculata* wings is the bright pterostigma, I predicted the pterostigma should be an important part of any display the female may perform and therefore the size and color may affect mating success. Although there was no connection between pterostigma size and mating frequency, the contrast between the light color of the pterostigma and the darker tip of the hindwing surrounding it was greater in females who mated compared to those that showed no evidence of mating. Greater contrast may increase visibility of females to males who are searching for mates, especially when the female is in dense vegetation or in flight. In order for the contrast between pterostigma and wing to be high, the wing must be darker, so wing pigmentation is also correlated with mating success. It is unclear whether males may select on the contrast itself or on the wing pigmentation alone. Of course, greater visibility to males also means greater visibility to predators. As mentioned earlier, forewings are more often associated with flight and therefore under selection pressures related to survival. There was relatively little variation in forewing size, however females that mated more often did show a pattern of larger forewings. The increased vulnerability to predation that comes

with greater contrast in hindwing pigment may require stronger flight abilities that come with larger forewings.

In the previous *Calopteryx* studies, there was evidence for selection on female wings, however the patterns were more often attributed to other mechanisms, including genetic correlation with males, in which females may have acquired certain traits because they inherited them from their fathers. For example, although Outomuro et al. (2012) found that both male and female hindwings of multiple *Calopteryx* species diverged before forewings, they concluded this was the result of sexual selection on the hindwings of males, but not females. However, they admit that although both sexes showed the same pattern of divergence, the shape of the hindwings is distinctly different in each sex (Johansson et al. 2009), thus it seems unlikely that the hindwing shape of females was the result of genetic correlation. Other suggestions for variation in female wings are that certain shapes may be better for oviposition (especially in species other than *C. maculata* that lay eggs beneath the surface of the water; Outomuro et al. 2012) and that wing ornamentation is important for species recognition (especially in sympatric species; Waage 1979; Svensson and Friberg 2007). While there is evidence for the validity of these explanations, it is clear that not all selective pressures on female wings have been investigated.

It has recently been acknowledged that while females may not perform sexual displays that are as overt or extreme as male displays, female behavior in general has been overlooked (Hosken & House 2011; Rosvall 2011). These observations have been made in multiple taxa, including red deer (Bebie & McElligott 2006), social lizards (While et al. 2009), dung beetles (Watson & Simmons 2010), dark-eyed Juncos, (Cain & Ketterson 2011), and house wrens (Krieg & Getty 2016). In *C. maculata*, I recorded female damselflies interacting

with each other and males in a way that was very similar to intrasexual male interactions.

This behavior is yet another trait, along with wing pigmentation, shape, and size, in which we see variation similar to what has been observed in males.

Conclusion

In the study of sexual selection on males, much of the variation we see in their reproductive success is due to the preferences of females for specific male traits and the result of male competition for access to females (Andersson 1994). We found variation in female mating success and differential reproductive success related to wing traits thought to be under selection in males only. These findings suggest that the same selection pressures may take place in females, and that females may also engage in intrasexual competition and be under male mate choice. Such patterns in females may be found to be widespread in nature, if studied in other systems. We understand much about how sexual selection acts on males, yet without equivalent study of reproductive trait variation within females, and thus the opportunity for selection to act on those traits, we cannot have a complete understanding of the role of sexual selection in the evolution of sexual traits in both sexes. The study of this variation is essential to understanding how evolution on females contribute to sex differences, and if such patterns are widespread we may need to reevaluate how we think about the role of females in sexual selection.

Appendix

Additional Tables and Figures from Methods and Results

Table A1. Distribution of powder colors on marked males showed no significant bias toward any color, $X^2 = 0.109$, $df = 3$, $P = 0.991$. Females were captured with all four colors of powder, indicating there was no preference for males marked with a particular color, $X^2 = 2.467$, $df = 3$, $P = 0.481$.

Powder color	Number of males marked 6/5 – 7/31/15	Number of females captured with powder color 6/6 – 8/2
Blue	230	135
Green	226	127
Orange	226	134
Pink	223	152

Table A2. Age distribution of all females as of first capture (6/6 - 8/2/15).

Age Category	Frequency
0.0	12
0.5	22
1.0	212
1.5	158
2.0	11
Total	415

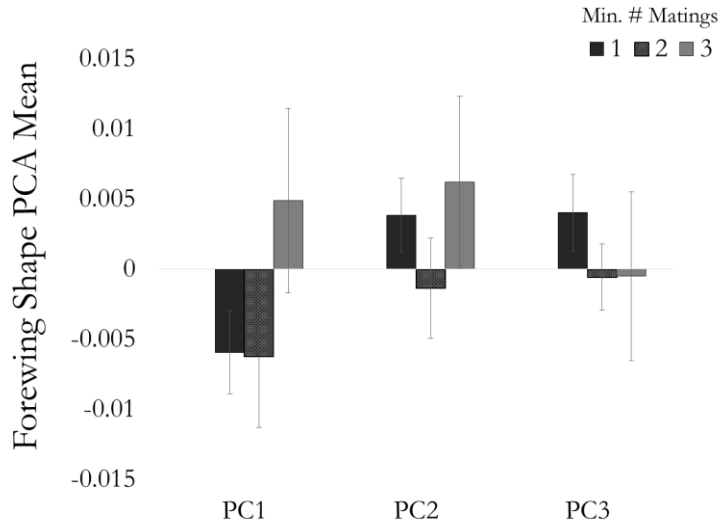


Fig. A1. There was no difference in mean PC1, PC2, and PC3 scores of forewing shape between mating groups, ANOVA, PC1: $F = 1.473$, $df = 3$, $P = 0.228$; PC2: $F = 0.3388$, $df = 3$, $P = 0.798$; PC3: $F = 0.539$, $df = 3$, $P = 0.657$.

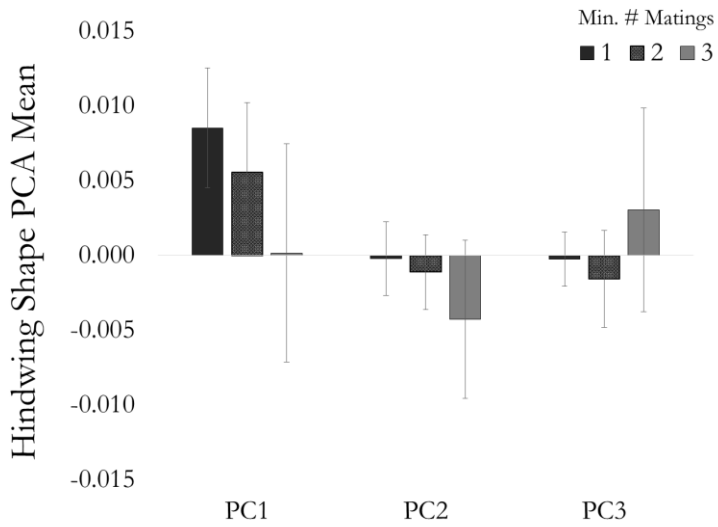


Fig. A2. There was no difference in mean PC1, PC2, and PC3 scores of hindwing shape between mating groups, ANOVA, PC1: $F = 1.651$, $df = 3$, $P = 0.184$; PC2: $F = 0.432$, $df = 3$, $P = 0.731$; PC3: $F = 0.398$, $df = 3$, $P = 0.755$.

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